ANTIBODY SEROLOGY — THE GOOD AND THE BAD

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Although a thorough physical examination and the careful use of ancillary testing (CBC, biochemistries, diagnostic imaging, etc.) enables the clinician to determine what disease process is occurring in a patient, determining the actual causative agent can sometimes remain elusive despite the clinician's best efforts. In those cases where determining an exact etiology is important—usually when an infectious agent is thought to be involved— further testing must be employed. This testing can be either direct (through the detection of antigen through DNA/PCR, histopathology, immunohistochemistry, or culture), or indirect (through the detection of specific antibodies to an antigen).

This antibody detection, known as serology, was originally designed to screen flocks to detect the presence of a disease. Today it is being used increasingly more commonly in individual birds, a purpose for which it was never designed. Although a valuable diagnostic tool, there are limits to its use which sometimes, unfortunately, clinicians do not always appreciate and can lead them to make an inappropriate diagnosis or institute an inappropriate treatment.

This paper seeks to guide clinicians in the use of serological tests through reviewing the avian humoral immune system, explaining the types of tests available, and outlining the different factors that can affect the results and their interpretation.

THE AVIAN HUMORAL IMMUNE SYSTEM — A QUICK REVIEW

The immune system serves two purposes: to clear infection from the body, and to develop a pathogen-specific resistance to protect from future infections. It has two layers of defense:

- Nonspecific pathways, responding to all foreign material. It includes mechanical barriers (the skin, mucosa, etc.) and the innate immune system (macrophages, heterophils, thrombocytes and complement). It gives the specific immune response time to develop; and
- Specific pathways, including cell-mediated immunity (T cells) and humoral immunity (B cells).

Humoral immunity is the process that is of interest in understanding serology. An antigen may activate a B-cell directly or initially bind to an accessory cell (macrophages, heterophils, and thrombocytes) that leads to the activation of T and then B cells. These B cells are produced in the bone marrow and mature as B lymphocytes in the bursa. They then differentiate into the antibody-producing plasma cells.

These antibodies react with a portion of the pathogen's surface (called the epitope) to maintain specificity. They coat extracellular pathogens, allowing the immune system to destroy them, while at the same time preventing the pathogen from entering target cells. (T cells, on the other hand, destroy cells that have already being infected or damaged.)

This antibody response can be either primary (the first time the body has been infected with that pathogen), or secondary (occurs with subsequent re-infections). A primary response may take 1 to 2 weeks to peak, as there is an exponential latent phase with a progressive increase in circulating antibody. Levels decline rapidly as other antibodies are produced by the immune system. A secondary response is much faster; the latent period is much shorter, and it usually produces more antibodies which last longer.

There is a difference between the antibodies produced by primary and secondary responses. A primary response produces IgM. This is a structurally large protein, so large that it is confined to the vasculature. After 1 to 2 weeks— and on subsequent re-infections—it is replaced by IgG. This is a smaller protein, capable of penetrating into tissue spaces.

Neonate chicks are not immunocompetent. Initially they rely on maternal immunity passed through the yolk and the albumen of the egg. The chick's immune system begins to function at around 2 weeks of age and maternal antibodies will be absent by 30 to 42 days. Most chicks begin to produce antibody at adult levels at around 6 weeks of age.

SEROLOGICAL TESTS

A variety of serological tests are available today. The selection of the best test requires a clinician to have an understanding of the principles of immunology and the interaction between host and pathogen. He/she also needs to have an understanding of the types of tests available and the advantages and disadvantages of each (see Table 1).

UNDERSTANDING THE USES AND LIMITATIONS OF SEROLOGY

Serology has several advantages that make it unlikely its use will ever disappear. First, PCR and other antigen-detection tests can sometimes be too accurate, detecting extremely low levels of antigen (or portions of antigen) that may not be significant. At other times the antigen may be extremely difficult to detect; it may shed only intermittently, or at very low concentrations. In both these cases, serological evaluation of the bird (or birds) may better detect the presence of significant levels of antigen. Serological assays of a flock may be more sensitive in detecting a pathogen than more direct antigen-detection tests.

A second advantage is its relatively low invasiness. Direct testing for an antigen that is only intermittently shed (if at all) often requires the collection of a tissue sample, either by autopsy or biopsy. Serology, on the other hand, simply requires a blood sample.

Although at first glance these appear to be almost overwhelming advantages, other factors play key roles in the selection of an appropriate serological test. These factors can be categorised into three groups:

The Characteristics of the Test

The **selection** of the test is of paramount importance. Is this test appropriate for this species? Has it been thoroughly validated for this disease? Antibodies are not necessarily consistent across species; a test that works well in poultry may not work well, or at all, in psittacines.

The **sensitivity** and **specificity** of a test will often determine its usefulness. Sensitivity is a measure of a test's ability to accurately detect antibodies in an infected bird. A highly sensitive test will hopefully detect most of the antibody-positive birds in a population. As such, it is useful as a screening test during the early days of a disease outbreak, where it is important to quickly identify potentially infected birds. Once these birds have been removed from a population, it becomes more important to be sure that the remaining birds that have tested negative are, in fact, not infected. At this time a highly specific test is desirable. Specificity is the proportion of truly noninfected birds that test negative. A clinician using a serological test should therefore be aware of the published sensitivity and specificity of a test.

Another test characteristic to be considered is its **complexity** to perform. The more complex a test is, the more susceptible it is to operator error, leading to erroneous results. Errors can occur in the collection, storing, and transport of the sample to the lab, and then again in carrying out the test. Equipment can be faulty, the operator may be inexperienced, or reagents may be out of date. There may also be error in the interpretation of the results, or even dissension about the significance of the results.

In some cases a test requires **specific reagents** which may not be readily available (e.g., the hemagglutination inhibition test for PBFD requires specially prepared galah erythrocytes, which are readily available in Australia, but harder to obtain elsewhere. [NB: goose erythrocytes can sometimes be used.]) This limits its usefulness in the USA, UK, and Europe.

The Characteristics of the Antigen and the Disease

Some antigens/diseases are not readily detectable by some tests due to the behavior of that antigen. Some viruses, for example, do not agglutinate erythrocytes, making hemagglutination inhibition tests unsuitable.

Some pathogens, such as viruses like the influenza A virus, undergo antigenic variance wherein the surface proteins of the virus change and prevent immediate detection. Other pathogens, such as *Mycobacterium* and *Aspergillus*, seem to not provoke an antibody response in some patients for, as yet, undetermined reasons. In these situations, false-negative test results may occur.

From an epidemiological point of view, there will always be false-positive and false-negative results for any test. The clinician must therefore interpret results with care; if a disease is of a low incidence in a species or in a geographical area, is a positive result truly significant or even accurate? And vice versa? As with any laboratory test, the results must be interpreted as a part of the diagnostic test, not as a sole entity.

Host Factors

Some clinicians seem to fall into the trap of forgetting that there is a patient attached to a laboratory test result. How the host is responding to the challenge of a pathogen has a major impact on the interpretation of a serological test (or any other test). The interaction between the host and the pathogen must be understood by the clinician in order to accurately assess the patient's status.

In the initial stages of infection (the pre-patent period) there will be a delay while the immune system first recognizes the presence of an antigen and then mounts a response to it (usually in the form of IgM). In this pre-patent period (usually 1–2 weeks), a serological assay that only detects IgG will give a false-negative result. On the other hand, direct antigen-detection tests may have a pre-patent period of only 1–2 days, and are therefore more reliable in detecting early infection.

With appropriate treatment or an effective immune defence (or both) the host will inactivate/kill the pathogen and then clear it from the body. During this process there is a rising antibody titer. After the pathogen has been cleared, these titres should start to decline. The rate of this decline is dependent on many factors: the strength of the immune system, the persistence of the antigen, and the degree of response to a specific pathogen. A single-point serological assay is unable to distinguish between rising and declining antibody titres; serial tests, 2–3 weeks apart, are needed to assess what is happening in the patient. For example, polyomavirus infection in a bird may result in detectable antibody titers for the life of the bird, even though the bird cleared the virus from its body after a few weeks or months. So while a single serological test may indirectly detect that a pathogen was/is present in the patient, it cannot (by itself) detect the ongoing presence of disease.

The serological detection of pathogens that are ubiquitous in the environment is fraught with error. Most birds, for example, are exposed to *Aspergillus spp* at some time or another; relatively few develop aspergillosis. The detection of antibodies to *Aspergillus* does not, therefore, constitute a diagnosis of aspergillosis; rather, it indicates the patient has been exposed to the pathogen and has mounted an immune response to it.

Despite the presence of a pathogen actively causing disease, some birds fail to produce antibodies. This immunosuppression can be due to a number of factors. Including concurrent disease, which frequently causes immunosuppression. Viruses such as PBFD, polyomavirus, pox, and herpes; bacterial or parasitic infections; and aflatoxicosis have all been implicated in immunosuppressed patients. Certain drugs, such as tetracycline, tylosin, and gentamicin, are known to decrease antibody production. Environmental stress, including inadequate temperatures, humidity, noise, and poor nutrition, is known to suppress both the bursa and

the thymus, possibly through corticosteroid production by the adrenal gland. Immunosuppressed birds, for whatever reason, may give a false-negative result.

A final consideration is that antibodies produced in response to a particular pathogen may be difficult to distinguish from antibodies produced against another pathogen. Antibodies produced by vaccination may be indistinguishable from those produced by natural infection. In either case, false-positive results can be produced.

THE USE OF SEROLOGY

Disease Outbreaks

Serology is often employed in the face of an outbreak of an infectious disease. Serology has the advantages of being relatively simple (most labs can perform it), quick, and cost-effective, particularly when compared with other (more direct) tests such as DNA/PCR and histopathology. Large numbers of birds can be tested quickly, giving an indication of the incidence of the disease in a given population. In the early stages of an outbreak, high sensitivity is needed; all infected (positive result) birds need to be identified as soon as possible. Sensitivity can be increased by using known high sensitivity tests; by increasing the sample size of the population being tested; and by parallel testing, using several different tests to increase the likelihood of an infected bird being detected.

Once the incidence of a disease has been determined, steps can be taken to control it. This will usually require the removal of birds that have tested positive and further testing of those that have tested negative. This introduces the problem of false-negative results— seronegative birds that are latent carriers of a disease. At this time highly specific tests are needed, and may require the employment of a different serological test or tests. As the incidence of a disease approaches zero, the detection of these latent carriers becomes even more difficult, and complete eradication may not be possible without complete culling.

Specific-Pathogen Free (SPF) Flocks

One of the cornerstones of successful farming is maintaining a disease-free flock. This is no different with birds, whether it is an intensive poultry flock, a zoological collection, or an avicultural facility. The development of a SPF flock requires the testing of all members of that flock, preferably before they join it.

Vets advising clients on a SPF protocol encounter similar problems to those faced by vets dealing with an outbreak of disease. No test can hope to be 100% sensitive and 100% specific. Therefore parallel testing (several different tests, not necessarily all serological) and serial testing (repeated testing over a period of time) may help to detect those birds that are latent carriers and test negative the first time. While this can add considerably to the cost of new birds, this must be weighed against the costs of remedying an outbreak of disease if it escapes detection and is introduced.

The Individual Bird

While serological tests are extremely useful in screening flocks, they often lack the sensitivity and specificity required for screening an individual bird. While relatively inexpensive, they often lack the accuracy of a more direct antigen-detection test. However, these more direct tests only detect the presence of a pathogen, not necessarily the presence of a disease.

Combining the two tests together can lead to a better screening of an individual. By looking for the pathogen, and the body's immune response to it, a better understanding of the bird's status can be achieved. When combined with ancillary tests such as hematology and biochemistry (which can reflect the effect the pathogen is having on the bird) a more complete picture of the host–pathogen interaction becomes apparent.

CONCLUSION

Serology— the detection of an antibody response to a pathogen— is commonly employed in avian medicine. Any clinician contemplating its use must have a good understanding of the principles of its use, particularly its shortcomings. But, when used in conjunction with antigen detection tests and other tests to assess the bird's response to disease, it provides a valuable tool in the detection and assessment of disease.

References and Recommended Reading

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Table 1. Serological Tests

Test	Technique	Disease/Pathogen	Advantages	Disadvantages
ELISA	Detection of antibody–antigen complex by use of a second enzyme-conjugated antibody that produces a color change	Adenovirus in poultry *Mycobacterium* *Chlamydophila* Paramyxoviruses *Aspergillus*	Simple to operate – rapid, automated	Requires species-specific second antibody
Hemagglutination Inhibition	Proteins present on the surface of some viruses cause agglutination of erythrocytes. By adding antibodies against these viral proteins, this agglutination is inhibited. Therefore if agglutination occurs, no virus-specific antibodies are present, and vice versa. Titers are determined by serial dilution of the sample.	Circovirus Paramyxoviruses Eastern Equine Encephalitis Avian Influenza	Secondary antibodies not required Inexpensive Good sensitivity	Serum must be treated to clear non-specific agglutination Not all avian viruses cause agglutination
Complement Fixation	Complement is needed to bind antibody and antigen. Detection of complement fixation demonstrates the presence of the antigen.	Polyomavirus Psittacine Herpesvirus *Mycobacterium* *Aspergillus* *Chlamydophila*	Quick, simple test Moderate sensitivity and specificity	Can have false positives Doesn't work in all species Usually only detects IgM

Table 1. (continued)

Virus Neutralization	Serum is mixed with an antigen. If antigen-specific antibody is present in the serum, the antigen will be neutralized, and will be incapable of causing cytopathic changes in a cell culture.	Polyomavirus Psittacine Herpesvirus	Very specific and sensitive Detects both IgM & IgG	Takes up to 7 days to run – requires both cell culture and virus propagation Nonspecific substances in the serum can give a positive result
Immunodiffusion assay	Antibody and antigen placed in agar gel diffuse towards each other. If an antibody-antigen complex forms, a precipitate develops that is visible as a white line.	Adenovirus in poultry Reovirus in psittacines	Simple and cheap Secondary antibodies not required Moderate specificity	Low sensitivity Difficult to quantify
Immuno-Fluorescent Antibody	Multiple dilutions of serum are incubated with cells infected with Ag fixed to plastic. A fluorescent-labeled second antibody is added. Positive results can be viewed with a fluorescent microscope	Avian Influenza *Chlamydophila*	Quick and simple Moderate sensitivity and specificity	Requires species specific second antibody Requires fluorescence microscope